REMARKS/ARGUMENTS

Claims 1-58 were pending. Claims 3, 4, 8-12, 19-43, 46-58 were withdrawn from further consideration as directed to non-elected subject matters in the present application before the amendment as set forth above. By this Amendment, claims 1, 2, 6, 7, 16, 44 and 45 are amended, claim 5 is canceled, and claims 13-15 are withdrawn.

In the Office Action mailed August 24, 2006, the Examiner maintained rejection of: (i) claims 1, 2, 5-7, 11, 16, 17, 44, and 45 under double patenting, (ii) claims 1, 2, 5-7, 8 and 16 under 35 U.S.C. §112, first paragraph, for lack of enablement, (iii) claims 1, 2, 7, 8 and 16 under 35 U.S.C. §112, first paragraph, for insufficient description, (iv) claims 1, 2, 5-7, 8, 16, 17, 18, 44 and 45 under 35 U.S.C. §103(a). The Examiner also asserted her position on the status of claims 13-15 as withdrawn, and objected to the specification, the drawings, and claim 44 for various reasons.

On October 24, 2006, Applicants filed a response to the Office Action of August 24, 2006 along with Amendment.

On November 17, 2006, the Examiner issued an Advisory Action indicating that the reply filed October 24, 2006 failed to put the application in condition for allowance.

Two issues were raised in the Advisory Action by the Examiner: (1) New claims 59 and 60 introduce new matter and require additional examination under USC 112 and 103; and (2) The Affidavit or other evidence filed after a final action will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented.

On December 11, 2006, Applicants communicated with the Examiner in an e-mail on the two issues raised in the Advisory Action as follows:

(1) New claims 59 and 60 are directed to the same subject matter as original claims 5 and 6, but with a scope being further limited (Claims 5, 6, 59 and 60 were attached in the Applicants' e-mail of December 11, 2006 for making comparisons). Moreover, the limitations recited in new claims 59 and 60 are extracted from the data that already exist in the tables disclosed in the specification as originally filed. Therefore, based on the standard stated under 35 U.S.C. 132 and

MPEP 706.03(o), the amendment does not introduce new matter into the disclosure of the invention.

(2) The scientific references cited in the Applicants' reply on October 24, 2006 were used to rebut the Examiner's assertions on the obviousness and her interpretations of the science described in the reference that she had referred to in her second Office Action.

Applicants' arguments convinced the Examiner to reconsider the application and on December 18, 2006, the Examiner communicated with Applicants by telephone and an e-mail along with an attachment of a proposed claim amendment. The Examiner suggested Applicants file a Second Response After Final and include arguments for: (1) "the relationship of thrombin's structure to its function has been well-characterized in the prior art," and provide some references. "Thus, the art provides guidance that would enable the skilled artisan to make and use the genus of thrombin variants having at least 80% homology to SEQ ID NO: 3;" (2) "that the dramatic extent of synergy upon mutation of the two sites overcomes the obviousness rejection. (see MPEP 716.02(c) below);" and (3) "file a Terminal Disclaimer to overcome the double patenting rejection."

Applicants very much appreciate the Examiner's professionalism and careful review of the present application, and in particular for the helpful guidance given to the Applicants.

In response, as set forth above, a Terminal Disclaimer has been submitted, claims 13-15 have been withdrawn, claim 5 has been canceled, and claims 1, 2, 6, 7, 16, 44 and 45 have been amended to conform to the Examiner's proposed claim amendment. Additionally, the specification has been amended according to the Examiner's suggestions in the Office Action of August 24, 2006.

Support for the amendments set forth above can be found in the disclosure as originally filed. Applicant asserts that no new matter is added.

It is now believed that amended claims 1, 2, 6, 7, 16, 44, 45, and pending claims 17-18 are in condition for allowance at least for the reasons set forth below and such allowance is respectfully requested.

The following remarks herein are considered to be responsive thereto.

Claim Set

In the Office Action mailed August 24, 2006, the Examiner asserted that "the status of Claims 13-15 as 'original' is incorrect. The status of said claims is withdrawn."

Applicants respectfully traverse the Examiner's assertion for the reasons that had been set forth in the prior response to the February 21, 2006 Office Action under the heading "Restriction Requirements." To advance the prosecution, however, Applicants have withdrawn claims 13-15 from consideration for now as set forth in the above claim amendments.

Specification Objections

The Examiner also objected to the specification because the June 20, 2006 amendment stating "the disclosure of which is hereby incorporated herein in its entirety by reference introduced New Matter to the specification."

In response, Applicants have amended the specification accordingly to overcome the objection.

Drawings Objections

The Examiner objected to Figs. 1-4 for "being confusing. Neither said figures nor the legends thereto explain the labeling found in figures."

Applicants have amended the Brief Description of the Figures to add legends to Figs. 1-4 explaining the labeling found in the figures. The support for the Amendment can be found in the specification, for examples, on page 13, lines 25-27. Accordingly, Applicants respectfully request that the objection to Figs. 1-4 be withdrawn.

Claim Objections

The Examiner objected to claim 44 "for being dependent from a non-elected claim."

Applicants have amended claim 44 to remove the dependency from a non-elected claim. Accordingly, Applicants respectfully request that the objection to claim 44 be withdrawn.

Claim Rejections Under Double Patenting

The Examiner maintained rejection of claims 1, 5-7, 16, 17, 44 and 45 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1, 5-7, 9, 10, 12 and 13 of the U.S. Patent 6,706,512. The Examiner also rejected claims 2 and 18 as being unpantable over claims 2 and 11, respectively, of the U.S. Patent 6,706,512 for the same reason.

Claim 5 has been canceled, which makes the obviousness-type double patenting rejection thereof moot.

As is well established (see MPEP § 804.02), a timely filed Terminal Disclaimer may be used to overcome such a rejection for each patent that is owned by the common assignee of the present patent application and each cited patent.

Accordingly, the undersigned attorney of record submit herewith Terminal Disclaimer on the behalf of Applicants of the present patent application and cited patent. The Terminal Disclaimer is directed to the following patent: U.S. Patent No. 6,706,512.

Claim Rejections – 35 USC § 112 First Paragraph

Enablement and Written Description:

The Examiner maintained rejection of claims 1, 5-7 and 16 under 35 U.S.C. §112, first paragraph, for lack of enablement, and rejected claims 2 and 8 on the same ground. Specifically, the Examiner asserted that "determining which of all polypeptides having at least 80% homology to SEQ ID NO: 3 have the desired activity would require undue experimentation."

The Examiner also maintained rejection of claims 1, 7, and 16 under 35 U.S.C. §112, first paragraph, for insufficient description, and rejected claims 2 and 8 on the same ground. Specifically, the Examiner asserted that "the polypeptides encompassed by the recited genus have any or no activity."

Claim 5 has been canceled, which makes the §112 rejection thereof moot.

Applicants respectfully traverse both the enablement and insufficient description rejections as ***the relationship of thrombin's structure to its function has been well-characterized in the prior art and thus, the art provides sufficient guidance that would enable***

the skilled artisan to make and use the thrombin variants having at least 80% homology to SEQ ID NO: 3 or 4.

For instance, Banfield et al. aligned the amino acid sequences of the nine vertebrate thrombin B chains with the previously published sequences of human and bovine thrombin B chins, and reported that "***[t]he overall amino acid sequence identity in this region of prothrombin is 43.3% between the 11 vertebrates; when conservative changes are included, the overall amino acid sequence similarity increases to 74.6%.***" *See* Banfield et al. Partial characterization of vertebrate prothrombin cDNAs: amplification and sequence analysis of the B chain of thrombin from nine different species. Proc. Natl. Acad. Sci 1992, Vol. 89, p 2779-2783 (Exhibit B, page 2782, left column, lines 10-14). The locations of insertion loops that interact with substrate, hirudin and thrombomodulin are also identified (*Supra*, Fig. 2, boxes A-D).

Moreover, studying evolution of prothrombin, Banfield et al. reported that chicken and hagfish prothrombin share 51.6% amino acid sequence identity, and both chicken and hagfish prothrombin are structurally very similar to human, bovine, rat, and mouse prothrombin and all six species share 41% amino acid sequence identify. *See* Banfield et al. Evolution of prothrombin: Isolation and characterization of the cDNAs encoding chicken and hagfish prothrombin. Journal of Molecular Evolution. 1994, Vol. 38, No. 2, p177-187. Abstract (Exhibit C).

Furthermore, Suzuki et al. published an article on "localization of thrombomodulin-binding site within human thrombin," which is relevant to the protein C activation activity of thrombin. *See* Suzuki et al. J Biol Chem. 1990, Vol. 5; 265(22):13263-7. Abstract (Exhibit D)

In addition, Petretski et al. discovered at least two forms of thrombin-like enzymes from snake *B. atrox*, which could transform mammalian fibrinogen into fibrin fibers, activate clotting factor V and plasma protein C, etc. These two forms of enzymes share 80% N-terminal sequence homology with the thrombin-like enzyme described for *B. moojeni*. *See* Petretski et al. Two related thrombin-like enzymes present in *Bothhrops atrox* venom. Braz J Med Biol Res, 2000, Vol. 33(11) 1293-1300. Introduction section (Exhibit E).

In particular, the amended claim 1 recites "wherein the variant thrombin has a PA/FC ratio greater than 1.0," which requires the proteins encompassed by the scope of the claimed invention have activity.

Since the relationship of thrombin's structure to its function has been well-characterized in the prior art, and the specification of the application further teaches the notion of PA/FC ration greater than 1.0, there is sufficient guidance that would enable the skilled artisan to make and use the invention claimed in claims 1, 2, 6, 7, 16-18, 44 and 45 at the time when the application was filed.

Accordingly, Applicants respectfully request the 35 U.S.C. §112, first paragraph, rejection under enablement and written description be withdrawn.

35 U.S.C. §103 Rejections

In the Office Action of August 24, 2006, the Examiner maintained rejection of claims 1, 5-7, 16, 17, 44 and 45 under 35 U.S.C. §103(a) as being unpatentable over Gibbs et al., 1996 in view of Arosio et al., 2000 or Ayala et al., 2001. The Examiner also rejected claims 2 and 18 as being unpatentable under 35 U.S.C. §103(a) as being unpatentable over Gibbs et al., 1996 in view of Arosio et al., 2000 or Ayala et al., 2001 for the same reason.

Claim 5 has been canceled, which makes the obviousness rejection thereof moot.

Applicants respectfully traverse the Examiner's obviousness rejection. MPEP §716.02(c) states that "***[e]vidence of unexpected results must be weighed against evidence supporting prima facie obviousness in making a final determination of the obviousness of the claimed invention.***" [Emphasis added]

In the instant case, the invention E217A/W215A possesses unexpected synergistic properties as shown in the attached Exhibit A. The dramatic extent of synergy upon mutation of the two sites overcomes the obviousness rejection. *See MPEP supra.*

Accordingly, the claimed invention is non-obvious over the Gibbs in view of Arosio. Therefore, Applicants respectfully request the 35 U.S.C. §103 rejection be withdrawn.

Replies to the Examiner's Assertions

In rebutting the Applicants' arguments for nonobviousness, the Examiner made the following assertions:

(A) Neither Gibbs et al. nor Arosio et al. are required to disclose a thrombin variant having both W215A and E217A, since this is a rejection under 35 U.S.C. 103(a).

(B) Arosio's "statement does not teach away from looking for additional single or multiple mutations that produce a thrombin variant that has even better anti-coagulant activity," or "teach away from making a double W215A+E217A mutant, or any other mutant," and "[m]oreover, Arosio et al. teach that further studies are necessary to identify more precisely the epitopes for protein C binding and that the penultimate ß-strand of thrombin's B chain, which includes residues 215-217, represents an important target for future mutagenesis studies."

(C) The "synergistic effect is not unexpected" and that "many enzymes have allosteric sites that act synergistically in both the activation and inhibition of the enzyme." Citing Metzler et al (2001).

(D) "The skilled artisan would know that it is the ratio of protein C activity to fibrinogen clotting activity (PC/PF), not the absolute protein C activity, that determines whether the action of thrombin will be primarily anti-coagulation, via the activation of protein C, or procoagulation, via cleavage of thrombin (Arosio et al, pg 8095, parg1)."

Before replying to the Examiner's aforementioned points (A)-(D), Applicants want to direct the Examiner's attention to the MPEP rules on the 35 U.S.C. 103 as it will be the basis of the Applicants' reply.

MPEP states that when applying 35 U.S.C. 103, "***the references*** must be considered as a whole and ***must suggest the desirability and thus the obviousness of making the combination***;" and "[t]he references must be viewed ***without the benefit of impermissible hindsight vision afforded by the claimed invention***." *See* §2141. (Emphasis added.)

MPEP further states that suggestion or motivation to modify the references must meet, among others, the following requirements:

I. ***The prior art must suggest the desirability of the claimed invention***;

II. The mere fact that references ***can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination***; and

III. A statement that modifications of the prior art to meet the claimed invention would have been "well within the ordinary skill of the art at the time the claimed invention was made"

because the references relied upon teach that all aspects of the claimed invention were individually known in the art is not sufficient to establish a prima facie case of obviousness without some objective reason to combine the teachings of the references. *See* §2143.01. (Emphasis added.)

Applying the MPEP rules, Applicants hereby address each point raised by the Examiner in the Final Office Action regarding the motivation to combine and the unexpected properties of the claimed invention as follows:

(Reply A): The claimed invention is directed to a variant thrombin comprising an amino acid sequence having the substitutions W215A and E217A. The invention E217A/W215A possesses unexpected synergistic properties as shown in the attached Exhibit A. ***Neither the primary reference nor the secondary reference provides the motivation to combine the references to do what the invention has done.***

In the First Office Action dated February 21, 2006, the Examiner asserted that:

> "[i]t would have been obvious to a person of ordinary skill in the art to combine the teachings of Gibbs with Arosio to prepare a thrombin variant comprising both W215A and E217A substitutions. ***Suggestion to do so is provided by Arosio et al.***, wherein they state that W215 and E217 are known to be important for thrombin function. Furthermore, ***suggestion and motivation to combine is based on the skilled artisan's desire to provide a thrombin variant with enhanced protein C activation and decreased fibrinogen cleavage.***" (Emphasis added.)

See First Office Action, page 13.

In the Final Office Action dated August 24, 2006, the Examiner shifted the basis of motivation after Applicants had responded by pointing out that the ***combination of E217A and W215A produced a decreased, rather than enhanced protein C activity.*** The Examiner then alleged that "the skilled artisan would know that each mutation produces a thrombin variant having enhanced anti-coagulation activity, [or enhanced ratio of protein C activity to fibrinogen clotting activity]." *See* Final Office Action, page 10.

Applicants respectfully submit that ***if the motivation to combine the two references were really that obvious as the Examiner alleged, the Examiner would have asserted the motivation based on the skilled artisan's desire to provide an enhanced PC/PF ratio at the first place, rather than alleged "the skilled artisan's desire to provide a thrombin variant with enhanced***

protein C activation." The Examiner would not have to scramble to calculate the PC/PF ratio in each cited reference after Applicants had brought the experimental results of the invention to the Examiner's attention in the prior June 20, 2006 response to the First Office Action.

Accordingly, even if "the skilled artisan had the ***desire to provide a thrombin variant with enhanced protein C activity and decreased fibrinogen cleavage,***" as alleged by the Examiner, the skilled artisan at the time the invention was made would not have understood that Arosio teaches, suggests, or provides a motivation to combine the W215A with the E217A to arrive at the claimed invention.

(Reply B): ***Arosio's statement that "further studies are necessary to identify more precisely the epitopes for protein C binding" cannot be a teaching or suggestion or providing a motivation*** to do this by introducing W215A into the primary reference's E217 ***because Arosio concluded that "the environment of W215 of thrombin is not significantly involved in the binding of protein C."*** Thus, ***skilled artisan in the field at the time the invention was made would not have understood Arosio's teaching as a suggestion to introduce W215A into E217A*** in order to identify more precisely the epitopes for protein C binding.

Further, Arosio's statement that "[f]uture studies are necessary to identify more precisely the epitopes for protein C and PAR-1 binding, and that [t]he penultimate ß-strand of the B chain hosts highly conserved residues such as W215 and G216 whose mutation affects both the specificity and catalytic activity of the enzyme, and that this region represents an important target for future mutagenesis studies" ***is not the same as teaching, suggesting or providing a motivation to further substitute E217 on the top of W215A,*** which is required by the present invention.

Also, Arosio's statement that "[t]he differential effect on binding of fibrinogen and protein C makes the W215A mutant the best anti-coagulant thrombin reported to date" ***is not the same as suggesting or teaching to further combine W215A with E217A.***

Furthermore, Arosio's statement that "the gain in anti-coagulant potency is larger [for the W215A mutant] than that of the E217 A mutant" ***is not the same as suggesting, teaching, or motivating to do W215A/E217A*** as the Applicants have done.

Moreover, obviousness cannot be based on mere speculation or conjecture. Arosio et al.'s ***explanation*** for the effects seen in the W215A mutants "that perturbation of residue 215

propagates to the neighbor residues G216 and E217, producing changes in the access to the S1 site and reduce Na+ binding" ***is not the same as teaching or suggesting or providing a motivation to the skilled artisan to further introducing the W215A into the E217 in order to make a double mutant E217/W215A.***

(Reply C): Applicants respectfully submit that the Examiner not only erred in making impermissible hindsight vision, but also erred in over-simplifying the complexity of cooperative changes in Enzymatic conformation here.

The life science/Biotechnology being in the area of unpredictable art, a synergistic effect cannot reasonably or necessarily be expected from allosteric sites. According to Metzler, cooperative phenomenon is more complex than many models having been proposed. *E.g. see* page 349, left column, second paragraph; and page 476, right column, last paragraph.

Allosteric interactions do not necessarily lead to synergy. Metzler does not teach that the allosteric sites of enzymes act synergistically in both the activation and inhibition of the enzyme. On the contrary, Metzler teaches that ***allosteric interactions "lead to cooperativity or anticooperativity in binding."*** (Emphasis added.). *See* Metzler, page 476, right column, third paragraph.

Even if given the benefit of the doubt that the allosteric interactions were ***only cooperative*** in Enzyme activity, ***the cooperativity is by no means synergy***. Metzler teaches that cooperative binding of substrates to enzymes is analogous to that of cooperative binding of oxygen by hemoglobin. McLennan reported that Hemoglobin has three ***allosteric sites***, and their ***interactions are non-synergistic but are simply additive.*** *See* attached Abstract (*Biochemistry and Molecular Biology International*, Vol. 44, No. 1, pages 175-183, 1998) (Exhibit F). Rao G.S. reported that Ascaris suumphosphofructokinase has two ***allosteric sites***, one for fructose 2,6-biphosphate and one for AMP, and that their effects on the enzyme ***are additive and not synergistic***. *See* attached Abstract (*Archives of Biochemistry and Biophysics*, Vol. 365, No. 2, pages 335-343(9), 1999) (Exhibit G).

The foregoing examples sufficiently demonstrate that the life science is unpredictable art and that results from experimental manipulations at the molecular or genetic level are not always obvious or predictable for skilled artisan in the field. Thus, the Examiner's assertions that allosteric sites act synergistically were not scientifically correct.

(Reply D): Having acknowledged (again, after the Applicants had pointed it out in the prior response mentioned above) that each of single mutant W215A (Arosio et al.; Table 1) and E217A (Gibbs et al; Table 1) had a decreased Protein C activity, the Examiner then alleged that "the skilled artisan would know that it is the ratio of protein C activity to fibrinogen clotting activity (PC/PF), not the absolute protein C activity, that determines whether the action of thrombin will be primarily anti-coagulation, via the activation of protein C, or procoagulation, via cleavage of thrombin (Arosio et al, pg 8095, parg1)."

Applicants respectfully submit that ***Arosio does not teach*** that "it is the ***ratio*** of protein C activity to fibrinogen clotting activity (PC/PF), not the absolute protein C activity, that determines whether the action of thrombin will be primarily anti-coagulation, via the activation of protein C, or procoagulation, via cleavage of thrombin." ***It is Applicants who teach the ratio of PA/FC in the specification.*** *See* the specification, page 16. Applicants respectfully submit that the Examiner was committing an error in making impermissible hindsight here.

Furthermore, ***the art does not teach it is the ratio PC/PF that determines*** whether the action of thrombin will be primarily anti-coagulation, via the activation of protein C, or procoagulation." ***The art teaches*** that Thrombin is an allosteric enzyme existing in two forms, slow and fast. The two forms are significantly populated in vivo, and the ***allosteric equilibrium*** can be affected by the binding of effectors and natural substrates. The fast form is procoagulant because it cleaves fibrinogen with higher specificity; the slow form is anticoagulant because it cleaves protein C with higher specificity. *See* Attached Dang et al. (Abstract, PNAS, Vol. 92, 5977-5981, 1955) (Exhibit H).

Thus, ***it is the allosteric equilibrium, or the ratio of specificity to cleave Protein C (i.e., slow form) to specificity to cleave fibrinogen (i.e., fast form), that decides whether the net effect of thrombin in vivo is aniticoagulation or procoagulation.***

Even if the Examiner's allegation "the skilled artisan would know that it is the ratio of protein C activity to fibrinogen clotting activity (PC/PF), not the absolute protein C activity, that determines whether the action of thrombin will be primarily anti-coagulation" were scientifically sound, the skilled artisan still would not know to combine references to arrive at the claimed invention because the cited references do not provide the motivation to do so.

Accordingly, the claimed invention is non-obvious over the Gibbs in view of Arosio. The unexpected synergistic results found by the Applicants should not be treated lightly and dismissed causally by the Examiner. Neither the primary reference nor the secondary reference provides the motivation to combine the references to do what the invention has done.

Thus, Applicants respectfully request that the obviousness rejection be withdrawn.

Any amendments to the claims not specifically referred to herein as being included for the purpose of distinguishing the claims from cited references are included for the purpose of clarification, consistence and/or grammatical correction only.

It is now believed that the application is in condition for allowance and such allowance is respectfully requested.

CONCLUSION

Applicants respectfully submit that the foregoing Amendment and Response place this application in condition for allowance. If the Examiner believes that there are any issues that can be resolved by a telephone conference, or that there are any informalities that can be corrected by an Examiner's amendment, please call the undersigned at 404-495-3678.

Respectfully submitted,
MORRIS, MANNING & MARTIN, LLP

January 18, 2007

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Exhibit A

The invention Shows Synergistic Results

(From Tables 1 and 2, *see* Specification, pages 48 and 50)

Property	E217A	W215A	WE
PA/FC *	40.06	170	2865
Fibrinogen k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.27	0.034	0.00089
Fibrin k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.15	0.053	0.0021
Protein C + TM k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.14	0.075	0.033
PAR1 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.66	1	0.026
Antithrombin III k_{on} ($\mu M^{-1}s^{-1}$)[d]	1	0.56	0.0040

*The PA/FC here are calculated from the data shown in Tables 1 and 2. The term "PA/FC ratio" as used herein refers to the ratio of the percent of wild-type protein C activation (PA) activity remaining in a thrombin variant relative to the percent of wild-type fibrinogen clotting (FC) activity remaining in the thrombin variant. A value of PA/FC greater than 1.0 indicates that the thrombin variant has reduced procoagulant fibrinogen cleavage activity relative to the residual anticoagulant activity resulting from protein C activation. *See* Specification 16.

Comparative Data Between Cited References and the Invention

Property	The Primary Reference E229A (E217A)	The Secondary Reference W215A	The Invention WE
PA/FC *	19.1	170	2865

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2779–2783, April 1992
Biochemistry

Partial characterization of vertebrate prothrombin cDNAs: Amplification and sequence analysis of the B chain of thrombin from nine different species

(blood coagulation/serine protease/thrombomodulin/polymerase chain reaction/protein evolution)

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Communicated by Earl W. Davie, September 20, 1991

ABSTRACT The cDNA sequence of the B chain of thrombin (EC 3.4.21.5) has been determined from nine vertebrate species (rat, mouse, rabbit, chicken, gecko, newt, rainbow trout, sturgeon, and hogfish). The amino acid sequence identities vary from 95.5% (rat vs. mouse) to 62.6% (newt vs. hogfish). Of the 249 amino acids spanned in all the species compared, there is identity at 110 (45.8%) positions. When conservative changes are included, the amino acid similarity increases to 75%. The most conserved portions of the B chain are the active-site residues and adjacent amino acids, the B loop, and the primary substrate-binding region. In addition, the Arg-Gly-Asp motif is conserved in 9 of the 11 species compared, and the chemotactic/growth factor domain is well conserved in all of the 11 species compared. The least conserved region of the B chain corresponds to surface loops, including the putative thrombomodulin-binding site and one of the hirudin-binding regions. The extent of the amino acid sequence similarity and the conservation of many of the functional/structural motifs suggests that, in addition to their role in blood coagulation, vertebrate thrombins may also play an important role in the general mechanisms of wound repair.

The final reaction of the coagulation pathway is the conversion of fibrinogen to fibrin by the serine protease thrombin (EC 3.4.21.5) (1, 2). In mammals, thrombin is generated from its zymogen, prothrombin, by the limited proteolytic action of factor Xa in the presence of factor Va, calcium ions, and phospholipid (2). In addition, thrombin also activates/inactivates other coagulation factors such as factor XIII, factors V and VIII, and protein C. The anticoagulant activity of thrombin is regulated through the interaction of thrombin with thrombomodulin (2, 3), an endothelial cell membrane protein related in structure to the low density lipoprotein receptor (4). While thrombomodulin likely binds to thrombin by an exposed surface loop, the precise binding site has yet to be resolved (5, 6). The thrombin/thrombomodulin complex activates protein C, which in the presence of protein S then degrades factors Va and VIIIa (2).

The enzymatic activity of thrombin is regulated by the endogenous protease inhibitor antithrombin III (2). Thrombin is also inhibited by protein inhibitors from nonplasma sources such as hirudin, from the European medicinal leech (7). The substrate specificity of thrombin is similar to that of trypsin, cleaving after basic amino acid residues (8). However, thrombin has a more restricted substrate range than trypsin. The molecular basis of this restricted substrate specificity is still unresolved but is thought to be the result of interactions of the substrates with secondary binding sites distant from the active site (9, 10).

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Thrombin is also a potent stimulator of tissue plasminogen activator release from endothelial cells (11), and various forms of thrombin are chemotactic for monocytes and neutrophils (12–14). This chemotactic activity is blocked by binding with hirudin or antithrombin III (15). For monocytes, the chemotactic activity has been localized to residues 334–399 of thrombin (16). In addition to having chemotactic activity, this polypeptide sequence induces differentiation of certain macrophage lines (17). Thrombin also contains an Arg-Gly-Asp tripeptide analogous to the adhesion site in adhesive proteins such as laminin, fibronectin, and fibrinogen and is a possible site through which thrombin binds to receptors (18). The fact that thrombin possesses these additional bioregulatory and growth-stimulating activities suggests it may also play an important role in the wound healing process as well as fibrinolysis.

Thrombin consists of two polypeptide chains joined by a disulfide bond. The A chain (49 amino acids in bovines and 36 in humans) has no known function. The B chain (259 amino acids in both humans and bovines) is structurally similar to other serine proteases (9, 10). When the amino acid sequence of the thrombin B chain is aligned with that of bovine chymotrypsin, amino acid sequence insertions in the B chain are found at exon junctions (19). The crystal structure of human α-thrombin has placed these amino acid insertions on the surface of the protein (20). Crystal structures have now been described for human α-thrombin (20) as well as the thrombin/hirudin complex (21, 22). The solution of the crystal structure of thrombin should facilitate the identification of regions on the surface of the protein that are potentially involved in protein–protein interactions.

Amino acid sequence data from homologous proteins in divergent species can provide valuable insight into the amino acid residues involved in active-site/substrate specificity, protein–protein interactions, and species-specific differences in biological processes. To examine the structural constraints during the evolution of prothrombin, we have used the polymerase chain reaction (PCR) (23, 24) to amplify and characterize approximately 900 base pairs (bp) of thrombin B-chain sequence from nine vertebrate species.†

MATERIALS AND METHODS

Sample Collection. Liver samples from rat (*Rattus norwegicus*), mouse (*Mus musculus*), rabbit (*Oryctolagus cuniculus*), chicken (*Gallus gallus*), gecko (*Gekko gekko*), Jap-

Abbreviation: UTS, untranslated sequence.

*To whom reprint requests should be addressed.

†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M81391 for *Gallus gallus*, M81392 for *Gekko gekko*, M81393 for *Eptatretus stouti*, M81394 for *Mus musculus*, M81395 for *Cynops pyrogastor*, M81396 for *Oryctolagus cuniculus*, M81397 for *Rattus norvegicus*, M81398 for *Oncorhynchus mykiss*, and M81399 for *Acipenser transmontanus*).

Table 1. Seven primers for amplification and sequencing of vertebrate thrombin B-chain cDNAs

Primer	Sequence	Amino acids spanned*
Th3	5′-CAGCTGCTGTGTGGGGCCAGCGTCATCAG-3′	347–355
Th4	5′-GCCTTGTAACCAGCACGAACAT-3′	502–508
Th7	5′-AGCGCACCTTGGCAGGTGATG-3′	333–340
Th10	5′-AAGGGCGTGTGACTCGC/ATCGCG-3′	458–465
Ser1†	5′-ACAAAAGCTTGA/AIGGICCICCIC/GT/AA/GTCICC-3′	526–532
His1†	5′-ACAGAATTCTCCGGTIC/CTIACIGCIGCICAC/TTG-3′	360–367
T_{17XSP}	5′-ACACTGCAGGAGCTCTCTAGATTTTTTTTTTTTTTTT-3′	

*Amino acid sequence numbering is based on human prothrombin (26).
†Adapted from ref. 28. The letter I in primers Ser1 and His1 represents deoxyinosine.

anese fire-bellied newt (*Cynops pyrogastor*), rainbow trout (*Oncorhynchus mykiss*), and sturgeon (*Acipenser transmontanus*) were generously provided by colleagues at the University of British Columbia. Hagfish (*Eptatretus stouti*) were purchased from Seacology (Vancouver).

RNA Isolation. Total cellular RNA was isolated from fresh or previously frozen liver samples by using the acid phenol extraction procedure as described by Chomczynski and Sacchi (25).

Oligonucleotide Preparation and Design. Oligonucleotides were synthesized on an Applied Biosystems model 380B or 391 DNA synthesizer. Primer sequences were selected after alignment of cDNA sequences from human (26), bovine (27), and chicken (unpublished data) prothrombin. A total of five oligonucleotides spanning approximately 240 amino acids of the B chain of thrombin were used for the amplification of all vertebrate cDNA fragments with the exception of hagfish prothrombin (see Table 1). Hagfish prothrombin cDNA fragments were amplified by using the degenerate inosine-containing primers Ser1 and His1 (28).

Preparation of Single-Stranded cDNA. Single-stranded cDNA was prepared as described (29) by using the oligonucleotide T_{17XSP} (see Table 1) as the primer. Reaction mixtures were incubated at 37°C for 1 hr, diluted to 200 μl with sterile distilled water, and stored at −20°C.

PCR. PCR reactions were performed as described (24), using 1–2 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus), and 2–5 ng of cDNA in 50-μl reaction mixtures.

Sequence Determination of Amplified cDNA Fragments. After amplification, PCR samples were made blunt-ended with the Klenow fragment of *Escherichia coli* DNA polymerase I (30). The amplified blunt-ended DNA was ligated into the *Hinc*II site of pUC19. A minimum of four independent PCR products were sequenced from each species examined.

Nucleic Acid and Amino Acid Sequence Analysis. Sequence data were analyzed with the Delaney DNA sequence program (Delaney Software, Vancouver). Amino acid sequences were aligned by using ESEE (31).

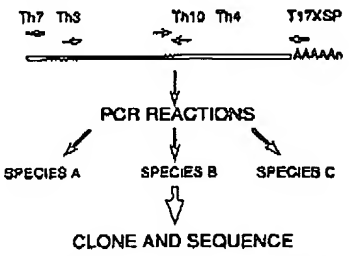

FIG. 1. Amplification strategy. The positions of the oligonucleotide primers used to amplify and sequence portions of the B-chain of vertebrate thrombin (see Table 1).

RESULTS

Amplification of Thrombin B-Chain cDNA Fragments. Amplification of single-stranded cDNA with primers Th7 and T_{17XSP} produced a fragment of ≈900 bp in all species examined except the hagfish (data not shown). Amplification of single-stranded cDNA with primers Th7 and Th4 produced a fragment 500 bp in length, and amplification with primers Th10 and T_{17XSP} yielded a fragment 300 bp in length (see Fig. 1). Amplification using the Ser1 and His1 primers produced fragments of ≈500 bp. This product represents a number of different trypsin-like serine protease cDNA fragments (28), including prothrombin (see Table 1).

Sequence Analysis of Amplified cDNA Fragments. With the exception of the fragments that were amplified by using the degenerate oligonucleotides, all amplified cDNA fragments contained a single species. Prothrombin cDNA fragments were identified by direct sequence analysis (32) and alignment of DNA and amino acid sequences with either human (26) or bovine (27) sequences. To identify the cDNA fragment corresponding to hagfish prothrombin, the Ser1 and His1 oligonucleotide primers were used. Several of the 500-bp fragments amplified by using these primers were cloned and their sequences were determined. Identification of the cDNA corresponding to the B chain of thrombin was based on the presence of amino acid residues Tyr-Pro-Pro-Trp. This sequence represents the B loop of thrombin (19). Among proteases, the B loop is unique to thrombin. The fragment corresponding to the B chain of hagfish prothrombin spanned the region from the active site histidine to the active site serine for a total of 161 amino acid residues.

On completion of sequence analysis, the portion of the B chain corresponding to residues 344–579 of prothrombin had been determined for each of the nine species. While the lengths of the coding sequences are similar in all the species examined (see below for the exceptions), the length of the 3′ untranslated sequence (UTS) is quite heterogeneous. The length of the 3′ UTS varies from 82 nucleotides in the trout and sturgeon to 1145 nucleotides in chicken, with the majority being ≈80–100 nucleotides (see Table 2). With the exception of the mouse and rat B chains, there are no significant regions of nucleotide sequence identity among any of the

Table 2. Length of thrombin B-chain coding sequences and 3′ UTS for the nine species

Species	Length, nucleotides	
	Coding sequence	3′ UTS
Rat	705	115
Mouse	705	114
Rabbit	705	121
Chicken	702	154/1145
Gecko	705	224
Newt	705	108
Rainbow trout	717	82
Sturgeon	702	82
Hagfish	708	407

species compared, excluding the polyadenylylation consensus sequence AATAAA. While this study was in progress the cDNA sequences of rat (33) and mouse (34) prothrombin were published. The sequences determined by PCR in this study are identical to these published sequences.

Amino Acid Sequence Alignments. The predicted amino acid sequences of the nine vertebrate thrombin B chains are aligned with the previously published sequences of human (26) and bovine (27) thrombin B chains in Fig. 2. The length of this portion of the B chain is relatively invariant; only a single amino acid insertion is present in some species at position 472 (Fig. 2, box D). The amino acid residue at position 472 is absent from chicken, newt, gecko, rainbow trout, sturgeon, and hagfish thrombin. In addition to the variability at position 472, there is variability in the composition and length of the C terminus (see Fig. 2). The amino acid sequences surrounding the active-site residues His-363, Asp-419, and Ser-525 are conserved in all 11 species (see Fig. 2). All of the eight tryptophan and seven cysteine residues seen in the human B-chain sequence are conserved in each of the species examined. Ten of the 12 prolines are also conserved. When the 240 amino acids of the B chain from the 11 different species are aligned, there is amino acid sequence variation at 130 positions (54%).

DISCUSSION

We have amplified, cloned, and determined the DNA sequences of the majority of the B chain of thrombin from nine different vertebrate species. Comparisons of amino acid sequences with those of previously characterized portions of human and bovine prothrombin suggest that thrombin is highly conserved throughout vertebrate evolution (see Table 3). Amino acid sequence identies range from 96.5% (between

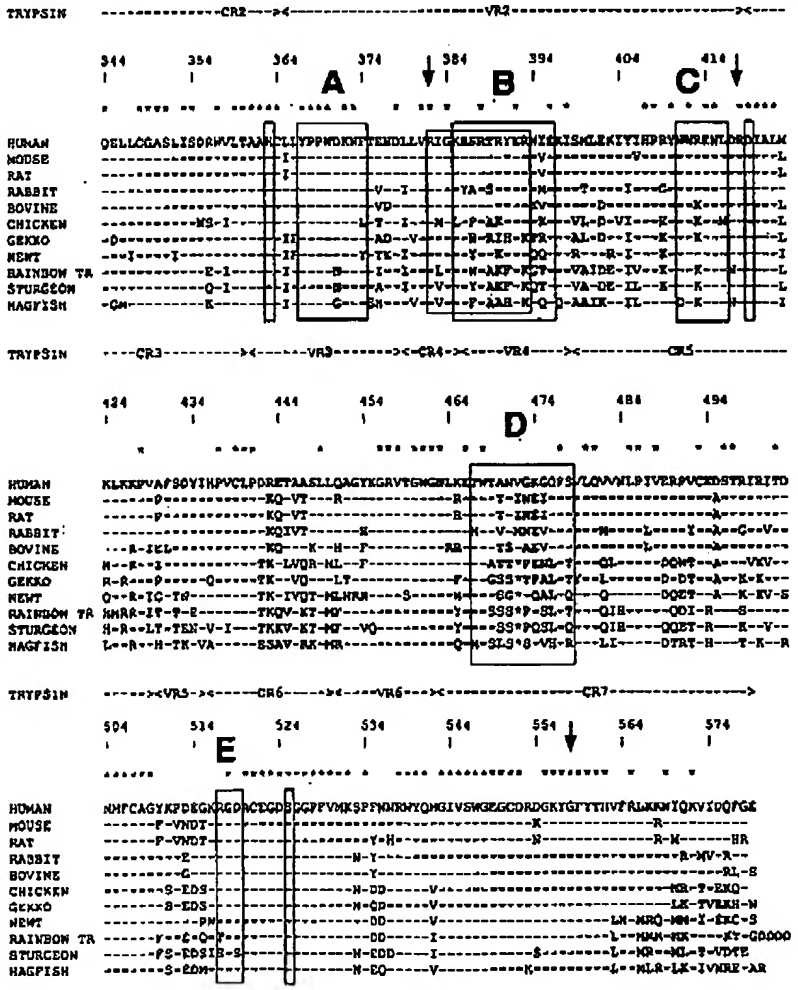

FIG. 2. Amino acid sequence alignment of vertebrate thrombin B-chain sequences. All of the aligned amino acid sequences were predicted from the translation products of the cDNA sequences. Amino acid sequence numbering is based on human prothrombin (26). Residues identical to the human sequence are indicated by a dash. The * above the human sequence identifies amino acid residues identical in all 11 species. Amino acid substitutions are as indicated, and the deletion at position 472 in some species is marked by an *. The active-site His, Asp, and Ser residues are marked by the narrow boxes. Arrows above the human sequence indicate the positions of Arg-382, Arg-418, and Gly-558 (see text for details). For descriptions of residues bounded by boxes A, B, C, D, and E, see text. The broken lines refer to positions representing either conserved regions (CR) or variable regions (VR) as described by Furie *et al.* (19).

Table 3. Percent amino acid sequence identity among vertebrate thrombin B-chains

	R	M	Rbt	B	C	G	N	RTr	S	Hgf
Human	88.5	89.8	85.1	87.3	72.2	72.8	70.2	68.6	65.0	65.3
Rat		96.5	82.6	86.0	71.4	73.2	68.5	68.5	65.5	63.8
Mouse			83.0	86.4	71.4	73.2	68.5	69.4	65.0	64.7
Rabbit				82.6	73.1	71.9	68.9	66.4	64.1	64.1
Bovine					72.2	72.3	68.5	68.8	65.0	64.0
Chicken						77.8	73.5	72.2	72.2	65.8
Gecko							71.1	70.2	70.1	69.4
Newt								69.4	69.2	62.6
Rainbow trout									82.5	68.6
Sturgeon										66.2

Percent identity data was generated with PALIGN (IntelliGenetics) using the structure-genetic matrix, open gap = 5; unit gap = 50. R, rat; M, mouse; Rbt, rabbit; B, bovine; C, chicken; G, gecko; N, newt; RTr, rainbow trout; S, sturgeon; Hgf, hagfish.

mouse and rat) to 62.6% (between newt and hagfish). Thrombins from mammalian species share greater than 82% amino acid sequence identity. The percent identity is much lower among the nonmammalian species (66% average). Rainbow trout and sturgeon share 82.5% amino acid sequence identity; this is 10% more identity than either shares with any of the other nine species. This high amino acid sequence identity may reflect a slower rate of nucleotide sequence change in fish or constraints on the B chain through interactions with other proteins involved in hemostasis. The overall amino acid sequence identity in this region of prothrombin is 43.3% between the 11 vertebrates; when conservative changes are included, the overall amino acid sequence similarity increases to 74.6%.

The specificity of thrombin for its substrates is likely determined by its insertion loops (10, 19, 20). The locations of these insertion loops are indicated in Fig. 2 and correspond to the VR regions defined by Furie *et al.* (19). A number of the loop segments are similar to those of chymotrypsin but contain additional residues, which alter the overall three-dimensional structure of the region (20). Several of the loop structures in the B chain of thrombin have been implicated in interactions with substrate (boxes A and C and the first four residues of box D in Fig. 2) and as sites of interaction with hirudin (box B in Fig. 2) (21, 22) and thrombomodulin (residues 382–392; see box B and box D in Fig. 2) (5, 6). With the exception of the B loop, corresponding to residues 365–369 (box A in Fig. 2), and the loop highlighted by box C (Fig. 2), all loop structures have highly variable amino acid sequences. This variability in surface loop amino acid sequence may contribute to some of the species-specific differences observed between thrombin and fibrinogen (35–37).

The human thrombin B chain contains a region with growth factor and chemotactic activity *in vitro* (16, 17). This chemotactic/growth factor domain corresponds to residues Leu-335 to Met-400 of prothrombin (see Fig. 2). In addition to the chemotactic/growth factor activity attributed to this region, residues 335–400 also contains the B loop (box A in Fig. 2), active-site histidine (His-363), a hirudin-binding loop (box B in Fig. 2), a potential thrombomodulin-binding site (Arg-382 to Arg-393; see Fig. 2), and a fibrinogen-binding site (6). With the exception of the hirudin binding site and the putative thrombomodulin-and fibrinogen-binding regions, this portion of thrombin contains very few amino acid sequence differences among the species examined. Analysis of the thrombin crystal structure has identified the B loop (box A in Fig. 2) as forming an extended loop structure that restricts access to the active-site cleft, which may be responsible for the limited substrate specificity of thrombin (20). Due to the rigid linked structure of the B loop, it has been suggested that this loop may also represent part of the active substructure of the chemotactic/growth factor domain (10, 20). The amino acid sequence similarity in this region (28/56 identical residues, 8/56 conservative changes) suggests that the chemotactic/growth factor activity attributed to this region in human thrombin may have a similar activity in other vertebrate thrombins. In addition to the putative role of the B loop in substrate specificity and chemotactic/growth factor activity, this portion of the B chain contains a site for N-linked glycosylation (Asn-373). The asparagine residue at position 373 is conserved in all of the 11 species.

Residues Lys-385 to Glu-396 (box B in Fig. 2) represent one of the surface contact loops of hirudin with human thrombin (21, 22). Interestingly, only 4 of the 12 residues in this region are identical or have conservative changes: Arg-388, Arg/Lys-390 (except hagfish and newt), Arg/Lys-393, and Lys-397 (except hagfish) (see box B in Fig. 2). Analysis of the crystal structure of the thrombin–hirudin complex reveals a number of possible electrostatic interactions between these residues and the acidic C-terminal residues of hirudin (21).

To date, two putative thrombomodulin-binding sites have been identified in human thrombin (5, 6). One binding site corresponds to residues Arg-382 to Arg-393 (see Fig. 2), a region of thrombin that has also been identified as binding hirudin (21, 22) and fibrinogen (6). The other thrombomodulin-binding site corresponds to residues Thr-468 to Ser-478 (box D in Fig. 2), where the surface loop formed by Glu-467 to Tyr-470 is located at the entrance of the binding cleft. The putative thrombomodulin-binding site at Arg-382 to Arg-393 (6) overlaps a hirudin-binding site (see box B in Fig. 2). The amino acid sequence of this region is conserved among the mammals compared, with the exception of the rabbit (see Fig. 2); all five of the basic residues in this region are conserved. The amino acid sequences of the nonmammalian species share an average of 50% amino acid sequence identity with the mammalian species. With the exception of the chicken sequence, 4/5 basic residues are conserved in the nonmammalian species compared. The putative thrombomodulin-binding site corresponding to residues Thr-468 to Ser-478 (box D in Fig. 2) (5) is highly variable, with only 2 of the 12 amino acid residues being invariant (Trp-468 and Pro-477). Unlike the putative thrombomodulin-binding site at Arg-382 to Arg-393, the amino acid sequence in this region varies significantly among the mammalian species compared. The majority of the amino acid sequence changes in this region are nonconservative. In addition, this is the only region of the B chain to contain an amino acid deletion. The precise location of the thrombomodulin-binding site(s) in human α-thrombin has yet to be resolved (5, 6, 38, 39).

Human thrombin contains an Arg-Gly-Asp tripeptide sequence at positions 517–519 (box E in Fig. 2) analogous to the adhesion site in adhesive proteins such as laminin, fibronectin, and fibrinogen (18). This region of thrombin has been shown to promote endothelial cell adhesion, spreading, and cytoskeletal reorganization, potentially contributing to repair mechanisms and maintenance of the internal blood vessel

lining (18). Although the cell adhesion activity was greatest with a chemically modified form of thrombin (NO_2-α-thrombin), native thrombin was also found to promote endothelial cell adhesion. While a portion of the loop segment Tyr-509 to Gly-518 is exposed to the solvent, the tripeptide Arg-Gly-Asp is not (20). The Arg-Gly-Asp sequence is conserved in 9 of the 11 species, supporting a possible role for this sequence in vertebrate thrombins *in vivo*.

The C-terminal region of thrombin is variable in both composition and length. Within the C-terminal 10 residues of human thrombin, only Trp-569 and Lys-572 are conserved in all of the species compared. According to the crystal structure, the C terminus of human thrombin is exposed to the solvent (20); however, no interactions or functions have been assigned to this region.

A number of abnormal human prothrombins have been characterized and their molecular defects have been identified. Three of these are found in the B chain. Prothrombin Quick I (40) is characterized by an Arg → Cys change at residue 382 (between boxes A and B in Fig. 2). The Arg residue is conserved in all 11 species. This amino acid has been identified from the three-dimensional structure as one of the residues lining the long groove extending from the active site and may form part of the putative fibrinogen secondary binding site (20). Substitution of Cys for Arg at this position probably disrupts thrombin/fibrinogen interactions. In prothrombin Tokushima, the Arg at position 418 is replaced by a Trp (41). This Arg residue is also conserved in all 11 species. Arg-418 is adjacent to box C (Fig. 2), which forms one of the surface loops projecting out from the active site cleft (20). It may be the nature of the amino acid change at position 418 that leads to the decreased enzyme efficacy observed in prothrombin Tokushima. A Gly → Val substitution at position 558 is found in prothrombin Quick II (42). Gly-558 is adjacent to the Cys-551 to Tyr-557 loop segment (20) and is conserved in all 11 species. Substitution of Val for Gly appears to alter the primary substrate-binding pocket (42).

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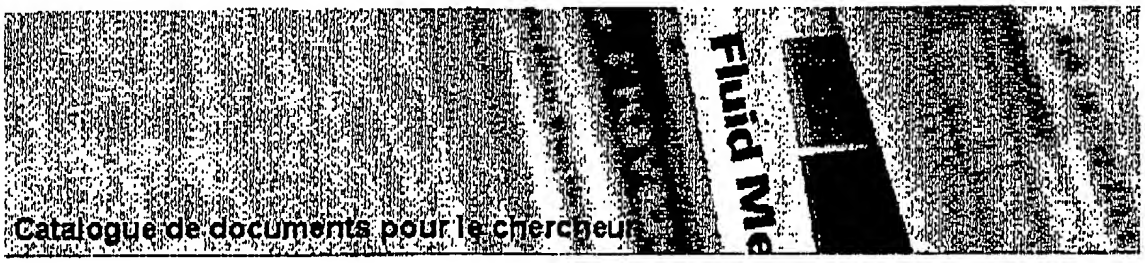
1. Jackson, C. M. & Nemerson, Y. (1980) *Annu. Rev. Biochem.* 49, 765–811.
2. Rosenberg, R. D. (1987) in *The Molecular Basis of Blood Diseases*, eds. Stamatoyannopoulos, G., Nienhuis, A. W., Leder, P. & Majerus, P. W. (Saunders, Philadelphia) pp. 534–574.
3. Jackman, R. W., Beeler, D. L., VanDeWater, L. & Rosenberg, R. D. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8834–8838.
4. Jackman, R. W., Beeler, D. L., Fritze, L., Soff, G. & Rosenberg, R. D. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6425–6429.
5. Suzuki, K., Nishioka, J. & Hayashi, T. (1990) *J. Biol. Chem.* 265, 13263–13267.
6. Noe, G., Hofsteenge, J., Ravelli, G. & Stone, S. R. (1988) *J. Biol. Chem.* 263, 11729–11735.
7. Markwardt, F. (1970) *Methods Enzymol.* 19, 924–932.
8. Fenton, J. W., II, (1981) *Ann. N.Y. Acad. Sci.* 370, 468–495.
9. Fenton, J. W., II, & Bing, D. H. (1986) *Semin. Thromb. Hemostasis* 12, 200–206.
10. Fenton, J. W. (1988) *Semin. Thromb. Hemostasis* 14, 234–240.
11. Levin, E. G., Stern, D. M., Nawroth, R. A., Marlar, R. A., Fair, D. S., Fenton, J. W., II, & Harker, L. A. (1986) *Thromb. Haemostasis* 55, 115–119.
12. Bar-Shavit, R., Kahn, A. J., Wilner, G. D. & Fenton, J. W., II (1983) *Science* 220, 728–731.
13. Bizios, R., Lau, L., Fenton, J. W., II, & Malik, A. B. (1986) *J. Cell Physiol.* 128, 485–490.
14. Morin, A., Arvier, M., Doutremepuich, C. & Vigneron, C. (1990) *Thromb. Res.* 60, 33–42.
15. Bar-Shavit, R., Bing, D. H., Kahn, A. J. & Wilner, G. D. (1985) in *Membrane Receptors and Cellular Regulation*, UCLA Symposium in Molecular and Cellular Biology, New Series, eds. Czech, M. P. & Kahn, C. P. (Liss, New York), Vol. 23, pp. 329–338.
16. Bar-Shavit, R., Kahn, A., Mudd, S., Wilner, G. D., Mann, K. G. & Fenton, J. W. (1984) *Biochemistry* 23, 400–403.
17. Bar-Shavit, R., Kahn, A., Mann, K. G. & Wilner, G. D. (1986) *Proc. Natl. Acad. Sci. USA* 83, 976–980.
18. Bar-Shavit, R., Sabbah, V., Lampugnani, M. G., Marchisio, P. C., Fenton, J. W., II, Vladavsky, I. & Dejana, E. (1991) *J. Cell Biol.* 112, 335–344.
19. Furie, B., Bing, D. H., Feldman, R. J., Robison, D. J., Burnier, J. P. & Furie, B. C. (1982) *J. Biol. Chem.* 257, 3875–3882.
20. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. T. & Hofsteenge, J. (1989) *EMBO J.* 8, 3467–3475.
21. Grutter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J. & Stone, S. R. (1990) *EMBO J.* 9, 2361–2365.
22. Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C. & Fenton, J. W., II (1990) *Science* 249, 277–280.
23. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* 230, 1350–1354.
24. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. (1988) *Science* 239, 487–491.
25. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
26. Degen, S. J. F., MacGillivray, R. T. A. & Davie, E. W. (1983) *Biochemistry* 22, 2087–2097.
27. MacGillivray, R. T. A. & Davie, E. W. (1984) *Biochemistry* 23, 1626–1634.
28. Sakanari, J. A., Staunton, C. E., Eakin, A. E., Craik, C. S. & McKerrow, J. H. (1989) *Proc. Natl. Acad. Sci. USA* 86, 4863–4867.
29. Frohman, M. A., Dush, M. K. & Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* 85, 8998–9002.
30. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 394–395.
31. Cabot, E. L. & Beckenbach, A. T. (1989) *Comput. Appl. Biosci.* 5, 233–234.
32. Gyllensten, U. B. & Erlich, H. A. (1988) *Proc. Natl. Acad. Sci. USA* 85, 7652–7656.
33. Dihanich, M. & Monard, D. (1990) *Nucleic Acids Res.* 18, 4251.
34. Friezner-Degen, S. J., Schaefer, L. A., Jamison, C. S., Grant, S. G., Fitzgibbon, J. J., Pai, J.-A., Chapman, V. M. & Elliot, R. W. (1990) *DNA Cell Biol.* 9, 487–498.
35. Doolittle, R. F. (1965) *Biochem. J.* 94, 735–741.
36. Doolittle, R. F., Oncley, J. L. & Surgenor, D. M. (1962) *J. Biol. Chem.* 237, 3123–3127.
37. Ratnoff, O. D. (1987) *Perspect. Biol. Med.* 31, 1–33.
38. Tsiang, T., Lentz, S. R., Dittman, W. A., Wen, D., Scarpati, E. & Sadler, J. E. (1990) *Biochemistry* 29, 10502–10512.
39. Hofsteenge, J., Braun, P. J. & Stone, S. R. (1988) *Biochemistry* 27, 2144–2151.
40. Henriksen, R. A. & Mann, K. G. (1988) *Biochemistry* 27, 9160–9165.
41. Miyata, T., Morita, T., Inomoto, T., Kawauchi, S., Shikakami, A. & Iwanaga, S. (1987) *Biochemistry* 26, 1117–1122.
42. Henriksen, R. A. & Mann, K. G. (1989) *Biochemistry* 28, 2078–2082.

Exhibit L



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Evolution of prothrombin : isolation and characterization of the cDNAs encoding chicken and hagfish prothrombin

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Résumé / Abstract
The cDNA sequences of chicken and hagfish prothrombin have been determined. The sequences predict that prothrombin from both species is synthesized as a prepro-protein consisting of a putative Gla domain, two kringle domains, and a two-chain protease domain. Chicken and hagfish prothrombin share 51.6% amino acid sequence identity (313/627 residues). Both chicken and hagfish prothrombin are structurally very similar to human, bovine, rat, and mouse prothrombin and all six species share 41%o amino acid sequence identity. Amino acid sequence alignments of human, bovine, rat, mouse, chicken, and hagfish prothrombin suggest that the thrombin B-chain and the propeptide-Gla domain are the regions most constrained for the common function(s) of vertebrate prothrombins

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Exhibit D

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1: J Biol Chem. 1990 Aug 5;265(22):13263-7.

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Localization of thrombomodulin-binding site within human thrombin.

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A binding site for thrombomodulin on human thrombin (alpha-thrombin) was elucidated by identifying an epitope for a monoclonal antibody for thrombin (MT-6) which inhibited the activation of protein C by the thrombin-thrombomodulin complex by directly inhibiting the binding of thrombin to thrombomodulin. An 8.5-kDa fragment isolated by digestion of thrombin with Staphylococcus aureus V8 protease followed by reversed-phase high performance liquid chromatography (HPLC) and a peptide isolated by reversed-phase HPLC after reduction of the 8.5-kDa fragment, which was composed of three peptides linked by disulfide-bonds, bound directly to MT-6 and thrombomodulin. The amino acid sequence of the peptide coincided with the sequence of residues Thr-147 to Asp-175 of the B-chain of thrombin. A synthetic peptide corresponding to Thr-147 to Ser-158 of the B-chain inhibited the binding of thrombin to thrombomodulin. Elastase-digested thrombin, which was cleaved between Ala-150 and Asn-151, lost its binding affinity for both MT-6 and thrombomodulin. These findings indicate that the binding site for thrombomodulin is located within the sequence between Thr-147 and Ser-158 of the B-chain.

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Exhibit E

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Two related thrombin-like enzymes present in *Bothrops atrox* venom

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Abstract

This article describes the presence of two new forms of a thrombin-like enzyme, both with apparent molecular masses of 38 kDa, in *Bothrops atrox* venom. Both share the ability to cleave fibrinogen into fibrin and to digest casein. Both present identical K_m on the substrate BA*p*NA.

Their N-terminal amino acid sequences are identical for 26 residues, sharing 80% homology with batroxobin and flavoxobin. Two groups of monoclonal antibodies (mAbs) raised against the purified enzyme forms recognized different epitopes of the putative corresponding enzymes present in *B. atrox* crude venom. On Western blotting analysis of *B. atrox* crude venom, mAbs 5DB2C8, 5AA10 and 5CF11, but not mAbs 6CC5 and 6AD2-G5, revealed two or more protein bands ranging from 25 to 38 kDa. By immunoprecipitation assays, the 6AD2-G5 mAb was able to precipitate protein bands of 36-38 kDa from *B. atrox*, *B. leucurus*, *B. pradoi*, *B. moojeni*, *B. jararaca* and *B. neuwiedii* crude venoms. Fibrinogen-clotting activity was inhibited when the

same venom specimens were pre-incubated with mAb 6AD2-G5, except for *B. jararaca* and *B. neuwiedii*.

Key words: venoms, *Bothrops atrox,* enzymes, thrombin-like enzymes, blood coagulation system

Introduction

The snake venom serine proteinase family includes enzymes that transform mammalian fibrinogen into fibrin fibers (1-3), activate clotting factor V (4,5) and plasma protein C (6,7), release kinins from kallikreinogen (8,9) and cleave the complement C3 component (10).

We have recently shown that the thrombin-like *Bothrops atrox* venom component from two different Amazon regions, i.e., Manaus and Tucuruí, presented distinct chromatographic profiles and capacity to clot fibrinogen. The thrombin-like activity was isolated as a 32-kDa protein which was recognized and neutralized by horse hyperimmune serum anti-*Bothrops* whole venom (11).

In this article, we present data indicating that highly purified *B. atrox* thrombin-like preparations contain at least two forms of the enzyme. Both clot purified bovine fibrinogen and hydrolyze a synthetic substrate, although exhibiting small differences in their molecular masses and in binding affinity for ion-exchange resins. Moreover, the enzymes share 80% N-terminal sequence homology with the thrombin-like enzyme described for *B. moojeni* (12). We also describe monoclonal antibodies able to recognize and efficiently neutralize the thrombin-like enzymes of the *B. atrox* snake group but not able to discriminate between the two forms of thrombin-like activity.

Material and Methods

Venoms, resins and reagents

Bothrops atrox venom was provided by the Laboratório de Herpetologia, Instituto Butantan, São Paulo, SP, Brazil. This snake colony was originally started with specimens captured in Tucurui, Pará, Brazil. Venom was filtered through a 0.45-µm membrane, lyophilized, divided into 10-mg aliquots and stored at -20°C.

Sephacryl S-100-HR and cyanogen bromide-activated Sepharose-4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and the HPLC column DEAE-5PW was purchased from BioRad Laboratories (Hercules, CA, USA). Bovine fibrinogen type IV, EDTA, and the synthetic substrates N(-benzoyl-L-arginine-*p*-nitroanilide (BA*p*NA), N-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The molecular weight markers illustrated in Figure 3 were 66 kDa (bovine serum albumin), 45 kDa (egg albumin), 29 kDa (carbonic anhydrase), 18 kDa (ß-lactoglobulin), and 14 kDa (lysozyme) from Sigma. The Mid-Range Protein Molecular Weight Markers from Promega Corporation (Madison, WI, USA) are listed in Figures 4 and 5.

Purification of fibrinogen-clotting activity from *B. atrox* venom

Venom samples of 50 mg dry weight were dissolved in 2.5 mM Tris-HCl buffer, pH 7.5, plus 150 mM NaCl and centrifuged to remove insoluble particles. The supernatant was applied to a Sephacryl S-100-HR (2.5 x 67 cm) column equilibrated with the same buffer. The flow rate was 1.3 ml/min and fractions of 2.5 ml were collected. Each fraction was assayed for fibrinogen-clotting activity and protein concentration was monitored at 280 nm. The fractions containing fibrinogen-clotting activity were pooled, concentrated by lyophilization and dialyzed against 2.5 mM Tris-HCl buffer, pH 7.5. The pooled activity was applied to an HPLC anion-exchange column (DEAE-5PW, BioRad) equilibrated with 2.5 mM Tris-HCl buffer, pH 7.5. Elution was performed with an NaCl gradient. The resulting fractions were assayed for fibrinogen-clotting activity and protein concentration was monitored at 280 nm. Two pools containing fibrinogen-clotting activity were obtained. The pools were named TLE1 and TLE2 (thrombin-like enzyme), concentrated by lyophilization, dialyzed against 2.5 mM Tris-HCl buffer, pH 7.5, and stored at -20°C.

Fibrinogen-clotting assay

Clotting time was determined by mixing 50 µl of the sample to be tested with 400 µl of purified bovine fibrinogen (2 mg/ml), 25 mM Tris-HCl buffer, pH 8.0, plus 150 mM NaCl at 25°C. A unit of fibrinogen-clotting enzyme was arbitrarily defined as the amount of enzyme preparation capable of coagulating the fibrinogen solution in 1 min.

Enzyme assays and determination of kinetic parameters

Thrombin-like enzyme activity on *p*-nitroanilide substrates was assayed in 50 mM Tris-HCl, pH 8.0, at room temperature. The reaction was performed in a volume of 0.5 ml and was started by adding the enzyme at a final concentration of 4.0 µg/ml. Substrate hydrolysis was monitored by measuring the increase in absorbance at 410 nm. For the determination of Michaelis constant (K_m) and catalytic constants (k_{cat}), at least 7 substrate concentrations were used. K_m and k_{cat} were calculated according to Aguiar et al. (13). The following *p*-nitroanilide substrates were used: BA*p*NA, N-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide and N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. To determine the effect of monoclonal antibodies on the enzyme activity, appropriate dilutions of each antibody were incubated with the enzyme preparations for 30 min in 50 mM Tris-HCl, pH 8.0, followed by the addition of 120 mM of substrate. BA*p*NA hydrolysis was recorded by the absorbance change at 410 nm. Inhibition activity was calculated on the basis of at least 5 experiments and is reported as percent.

N-terminal sequence determination

N-terminal sequences of the purified proteins were analyzed on a Shimadzu PPSQ-10 Automated Protein Sequencer by Edman degradation. PTH-amino acids were detected at 269 nm after separation on a reverse phase C18 column (4.6 x 250 mm) under isocratic conditions according to manufacturer instructions. Sequence homology and alignments were determined using the BLAST NCBI algorithm (14).

Monoclonal antibody (mAb) production

BALB/c mice were immunized with TLE2 both before (mAbs: 6AD2-G5, 6CC5) and after SDS-

PAGE separation (mAbs: 5BA9, 5DB2C8, 5AA10, 5CF11, 5DC2) using $Al(OH)_3$ as adjuvant. Sixteen days later a booster injection was applied with the corresponding antigens without adjuvant. Spleen cells were harvested on day 30 and fused with NSO myeloma cells according to Köhler and Milstein (15). The supernatants of hybrid cells were screened by ELISA or Western blot methods. These supernatants were also screened for the ability of mAbs to block the fibrinogen-clotting activity present in total *B. atrox* venom or in purified thrombin-like preparations.

Immunoprecipitation

Twenty mg of CNBr-activated Sepharose-4B was equilibrated with 0.5 M HCl and 0.5 M NaCl, pH 2.0, for 30 min. After washing the gel with 100 mM carbonate buffer, pH 9.0, containing 0.5 M NaCl, 20 mg of mAb was added. The mixture was then incubated under gentle shaking for 4 h at room temperature, followed by 16 h at 4°C. The gel was washed with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, 0.1% sodium azide and 0.1% Nonidet P40, followed by the addition of the antigen. The mixture was incubated at 37°C for 1 h. The gel was washed with the same buffer, centrifuged and resuspended in 50 µl of SDS-PAGE sample buffer, boiled at 100°C for 10 min, and applied to a 12% polyacrylamide gel. Protein bands were detected by Coomassie blue.

Results

B. atrox venom was fractionated on Sephacryl S-100-HR and the fibrinogen-clotting activity was assayed. The fibrinogen-clotting activity reached its maximum at the elution volume of 190 ml (Figure 1A), in a region where the caseinolytic hydrolysis was low (Figure 1B), suggesting that the observed fibrinogen-clotting activity is a specific proteolytic activity not able to hydrolyze casein.

Fractions containing more than 10 units/ml of fibrinogen-clotting activity were pooled (Sephacryl pool), concentrated and applied to a DEAE-5PW HPLC anion-exchange column eluted with an NaCl gradient. Two protein peaks presenting fibrinogen-clotting activity were eluted and denoted TLE1 and TLE2, as indicated in Figure 2. Table 1 shows a summary of the purification steps, resulting in an enrichment of 37-fold with 7% recovery of activity in relation to crude venom.

The electrophoretic profile of *B. atrox* crude venom and TLE1 and TLE2 after purification is shown in Figure 3. The TLE1 and TLE2 proteins resulting from the ion-exchange step appear as bands of 38 kDa. Computed densitometric analysis (16) was used to estimate the extent of homogeneity as 95% for both proteins, indicating the existence of minor contaminants with a molecular mass of about 30 to 32 kDa.

Table 2 shows that both enzymes presented a similar kinetic behavior. TLE1 and TLE2 K_m for BA*p*NA were not significantly different, and both enzymes failed to hydrolyze efficiently the other substrates used. Among the synthetic substrates used to assay the enzymatic activities of TLE1 and TLE2, BA*p*NA was the one most rapidly hydrolyzed (Table 2) and was used in the inhibition experiments. The enzymatic activity of TLE1 was significantly blocked by 1 mM PMSF (28% inhibition) and by 10 µg of the mAb 6AD2-G5 (53% inhibition). The enzyme was

insensitive to 0.001 mM pepstatin, 10 mM EDTA and 0.01 mM E-64 (data not shown). When tested with the same inhibitors, TLE2 presented identical behavior, supporting the conclusion that TLE1 and TLE2 are serine proteinases.

The first 26 N-terminal amino acid residues of both TLE1 and TLE2 are identical (Table 3). When compared with published sequences for related proteins, homology was observed to be around 80% with batroxobin (12) and flavoxobin (17).

The protein profile of *B. atrox* crude venom was visualized with the mAbs against purified TLE2 by Western blotting (Figure 4). The mAbs 5BA9 (lane 1) and 5DB2C8 (lane 2) revealed strong protein bands with molecular masses of around 32 to 38 kDa. The apparent thickness of the bands suggests more than one protein component. The mAb 5AA10 (lane 3) revealed two faint protein bands, with molecular masses of about 35 and 25 kDa, respectively; mAb 5CF11 (lane 4) obtained two distinct but close protein bands with molecular masses of 38 kDa; mAb 5DC2 (lane 5) reacted with four distinct protein bands with molecular masses ranging from 26 to 38 kDa; mAbs 6CC5 (lane 6) and 6AD2-G5 (lane 7) were unable to react with protein bands within the 25 to 39 kDa range of molecular mass, although a faint band with a molecular mass lower than 14 kDa was observed in lane 6. The unrelated anti-human heat shock protein HSP70 mAb (lane 8) did not react with any *B. atrox* venom protein band.

The apparent inability of mAb 6AD2-G5 (Figure 4, lane 7) to react with electrotransferred crude venom proteins could be attributed to the denaturating conditions used in Western blotting. We hypothesize that mAb 6AD2-G5 may be directed at a conformational rather than a structural epitope. If this were the case, its conformation could be disarranged during the Western blotting procedure. To test this hypothesis, mAb 6AD2-G5 was immobilized on CNBr Sepharose-4B and used to immunoprecipitate the specific antigens present in whole *B. atrox* venom. Under these conditions, the epitope recognized by mAb 6AD2-G5 was preserved and 36-38-kDa protein bands were detected in *B. leucurus*, *B. pradoi*, *B. jararaca*, *B. neuwiedii*, *B. moojeni*, and *B. atrox* venom (Figure 5). The fibrinogen-clotting activity present in these venoms, but not in *B. jararaca* or *B. neuwiedii* venoms, was extensively blocked when samples were pre-incubated with mAb 6AD2-G5 (Table 4).

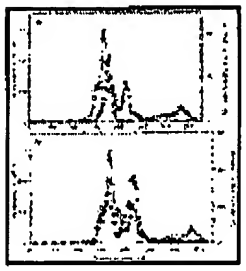

Figure 1 - Size exclusion chromatography of crude *B. atrox* venom (50 mg) on a Sephacryl S-100-HR column (2.5 x 67.0 cm). The venom was solubilized in 25 mM Tris-HCl, pH 7.5, plus 150 mM NaCl. Protein concentration was monitored at 280 nm (circles). *A*, Thrombin-like activity is indicated by triangles. *B*, Caseinolytic activity is indicated by triangles.

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Figure 2 - Ion-exchange chromatography. The gel-filtered thrombin-like activity (Figure 1A) was applied to a DEAE-5PW (BioRad) HPLC column, equilibrated with 2.5 mM Tris-HCl, pH 7.5, and eluted with an

NaCl gradient. Absorbance was monitored at 280 nm. Thrombin-like activity (filled circles) was estimated as described in Material and Methods. Peaks 1 and 2 were pooled as indicated by bars and are denoted TLE1 and TLE2.

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Figure 3 - SDS-polyacrylamide gel electrophoresis of purified thrombin-like enzymes (TLE) from *Bothrops atrox* venom. Samples were submitted to electrophoresis on 12% slab gel containing 1% SDS. Proteins were denaturated by heating. *Lane 1*, 60 µg of crude venom. *Lane 2*, 1 µg of TLE1. *Lane 3*, 1 µg of TLE2.

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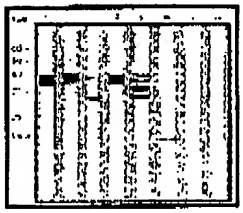

Figure 4 - Western blotting analysis using mAbs against *B. atrox* thrombin-like enzymes. Crude venom was submitted to SDS-PAGE (12% acrylamide), electrotransferred to a nitrocellulose membrane and each strip was reacted with mAbs as described in Material and Methods. *Lane 1*, 5BA9; *lane 2*, 5DB2C8; *lane 3*, 5AA10; *lane 4*, 5CF11; *lane 5*, 5DC2; *lane 6*, 6CC5; *lane 7*, 6AD2-G5; *lane 8*, anti-human HSP70 mAb.

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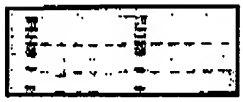

Figure 5 - SDS-PAGE (12% acrylamide) of immunoprecipitated venom proteins of *Bothrops* species by 6AD2-G5 immobilized on Sepharose-4B. Bands close to the 55,000 and 31,000 markers, present in all slots, are the heavy and light IgG fractions. The amounts of venom used in the immunoprecipitation experiments are given in parentheses. *Lane 1*, *B. fonsecai* (0.41 mg); *lane 2*, *B. leucurus* (0.58 mg); *lane 3*, *B. pradoi* (0.41 mg); *lane 4*, *B. jararaca* (0.54 mg); *lane 5*, *B. insularis* (0.28 mg); *lane 6*, *B. cotiara* (0.25 mg); *lane 7*, *B. alternatus* (0.33 mg); *lane 8*, *B. neuwiedii* (0.33 mg); *lane 9*, *B. jararacussu* (0.33 mg); *lane 10*, *B. moojeni* (0.30 mg); *lane 11*, *B. atrox* (0.64 mg).

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Discussion

Edema and pain are the earliest local symptoms following injection of *Bothrops* venom into the victim's skin. Thereafter, leukocyte infiltration, hemorrhage and intravascular thrombus formation may sometimes lead to severe tissue destruction (18). Hypotension, blood incoagulability with intense fibrinogen consumption and respiratory distress are concurrent systemic symptoms. Depending on the amount of venom injected, kidney failure and death are possible outcomes (19).

The snake venom serum proteases through their fibrinogen-converting activities (2), blood clotting factor V and protein C-activating enzymes (4-6), and C3-converting enzymes (10) are probably the most important mediator inducers of the tissue damage and symptoms provoked by *Bothrops* venoms.

The purification and characterization of two new forms of thrombin-like enzyme were reported here. Fibrinogen-clotting activities were concentrated into two closely related fractions, called TLE1 and TLE2, after serial chromatography on Sephacryl S-100-HR and DEAE-5PW. These fractions together, although representing only 7% of the total activity contained in the starting crude venom, have high specific activity (3100 U/mg) corresponding to 37 times purification (Table 1). Both enzymes are almost equally active on synthetic specific substrates such as BA*p*NA, suggesting that their active enzymatic sites are functionally similar. The capacity of TLE1 to hydrolyze the synthetic substrate was efficiently inhibited by mAb 6AD2-G5.

The purified proteins have similar molecular masses of 38 kDa (Figure 3). When amounts above 10 µg of TLE1 or TLE2 are submitted to SDS-PAGE (15%), a second band with a molecular mass of approximately 32 kDa becomes visible probably corresponding to the thrombin-like enzyme previously described by Cavinato et al. (11). These data, and the distinct elution profiles exhibited by the two enzymes (TLE1 and TLE2) on DEAE-5PW ion-exchange chromatography strongly suggest that although they contain similar enzymatic active sites they are distinct molecules. As the amino acid sequencing data presented in this paper did not extend beyond residue 26, differences in the primary structure could be present beyond this residue. Alternatively, the molecular differences between the two enzymes could be ascribed to the extent of glycosylation of their respective polypeptide chains. This possibility is likely since thrombin-like enzymes exhibiting microheterogeneity due to differences in their polypeptide chain glycosylation have been reported in venoms from *Agkistrodon rhodostoma* (20,21) *Crotalus horridus horridus* (22), *Lachesis muta muta* (23), *Bothrops jararaca* (24) and *Bothrops jararacussu* (25).

The N-terminal sequences of the TLE1 and TLE2 proteins have a high homology, around 80%, compared to published sequences for related proteins such as batroxobin and flavoxobin (12,19).

Since most assayed mAbs were able to recognize and precipitate protein components present in *B. atrox* crude venom, it was interesting to determine if they could also block the thrombin-like component against which they were raised. In preliminary experiments, we found that such activity was exhibited only by mAbs 5DB2C8 and 6AD2-G5. The latter mAb significantly blocked the fibrinogen-clotting activity present in *B. atrox* (80% inhibition), *B. leucurus* (90% inhibition), *B. moojeni* (84% inhibition), and *B. pradoi* (94% inhibition), as well as the purified thrombin-like activity of *B. atrox* venom.

The ability of mAb 6AD2-G5 to efficiently neutralize the fibrinogen-clotting activity only of venoms from *B. atrox*, *B. leucurus*, *B. pradoi* and *B. moojeni* supports the view that these species are highly correlated and distinct from the other *Bothrops* species (26). On the other hand, the

mAb was also able to recognize and inhibit with lower efficiency fibrinogen-clotting enzymes from *B. jararaca* and *B. neuwiedii* venoms. This mAb may be a valuable tool for the rapid purification of sufficient amounts of these proteins to be used in more refined experiments.

References

1. Stocker K & Meier J (1988). Thrombin-like snake venom enzymes. In: Pirkle H & Markland FS (Editors), *Hemostasis and Animal Venoms*. Marcel Dekker, New York.

2. Pirkle H & Theodor I (1991). Thrombin-like enzymes from snake venoms. In: Tu AT (Editor), *Handbook of Natural Toxins. Vol. 5. Reptile Venoms and Toxins*. Pergamon Press, London.

3. Zhang Y, Gao R, Lee WH, Zhu SW, Xiong YL & Wang WY (1998). Characterization of a fibrinogen-clotting enzyme from *Trimeresurus stejnegeri* venom and comparative study with other venom proteases. *Toxicon*, 36: 131-142.
[Medline]

4. Kisiel W (1979). Molecular properties of the factor V-activating enzyme from Russell's viper venom. *Journal of Biological Chemistry*, 254: 12230-12234.
[Medline]

5. Tokunaga F, Nagasawa K, Tamura S, Miyata T, Iwanaga S & Kisiel W (1988). The factor V-activating enzyme (RVV-V) from Russell's viper venom. Identification of isoproteins RVV-Va, -Vb and -Vr and their complete amino acid sequence. *Journal of Biological Chemistry*, 263: 17471-17481.
[Medline]

6. Kisiel W, Kondo S, Smith KJ, McMullen BA & Smith LF (1987). Characterization of a protein C activator from *Agkistrodon contortrix contortrix* venom. *Journal of Biological Chemistry*, 262: 12607-12613.
[Medline]

7. McMullen BA, Fujikawa K & Kisiel W (1989). Primary structure of a protein C activator from *Agkistrodon contortrix contortrix* venom. *Biochemistry*, 28: 674-679.
[Medline]

8. Rocha e Silva M, Beraldo WT & Rosenfeld G (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and trypsin. *American Journal of Physiology*, 156: 261-271.

9. Markland FS, Kettner C, Schiffman S, Shaw E, Bajwa SS, Reddy KN, Kirakossian H, Patkos GB, Theodor I & Pirkle H (1982). Kallikrein-like activity of crotalase, a snake venom enzyme that clots fibrinogen. *Proceedings of the National Academy of Sciences, USA*, 79: 1688-1692.

10. Tambourgi DV, dos Santos MC, Furtado NFD, Freitas NCW, Dias da Silva W & Kipnis TL (1994). Pro-inflammatory activities in Elapidae snake venoms. *British Journal of Pharmacology*, 112: 723-727.

11. Cavinato RA, Remold H & Kipnis TL (1998). Purification and characterization of thrombin like activity from the venom of *Bothrops atrox* of different geographic regions. *Toxicon*, 36: 257-268.

12. Itoh N, Tanaka N, Mihashi S & Yamashima I (1987). Molecular cloning and sequence analysis of cDNA for batroxobin, a thrombin-like snake venom enzyme. *Journal of Biological Chemistry*, 262: 3132-3135.
[Medline]

13. Aguiar AS, Alves CR, Melgarejo A & Giovanni-De-Simone S (1995). Purification and partial characterization of a thrombin-like gyroxin enzyme from bushmaster (*Lachesis muta rhombeata*) venom. *Toxicon*, 34: 555-565.

14. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25: 3389-3402.
[Medline]

15. Köhler J & Milstein C (1975). Continuous culture of fused cells secreting antibody of predefined specificity. *Nature*, 256: 495-497.

16. Bozzo S & Retamal C (1991). Gel-perfect: Geles unidimensionales, un nuevo método densitométrico para computadores personales. *Archivos de Biologia y Medicina Experimentales*, 24: 181-184.

17. Shieh TC, Kawahata SI, Kihara H, Ohno M & Iwanaga S (1988). Amino acid sequence of a coagulant enzyme, flavoxobin, from *Trimeresurus flavoviridis* venom. *Journal of Biological Chemistry*, 103: 596-605.

18. Rosenfeld G (1971). Symptomatology, pathology and treatment of snake bites in South America. In: Bucherl W, Buckley E & Deulofeu V (Editors), *Venomous Animals and Their Venoms*. Academic Press, New York.

19. Kamiguti AS & Cardoso JLC (1989). Haemostatic changes caused by the venoms of South American snakes. *Toxicon*, 27: 955-963.
[Medline]

20. Hatton MWC (1973). Studies on the coagulant enzyme from *Agkistrodon rhodostoma* venom. Isolation and some properties of the enzyme. *Biochemical Journal*, 131: 799-807.
[Medline]

21. Burkhart W, Smith GF, Su JL, Parikh I & LeVine H (1992). Amino acid sequence determination of ankrod, the thrombin-like α-fibrinogenase from the venom of *Agkistrodon rhodostoma*. *FEBS Letters*, 297: 297-301.

22. Shu YY, Moran JB & Green CR (1983). A thrombin-like enzyme from rattlesnake venom. *Biochimica et Biophysica Acta*, 748: 236-244.

23. Silveira AMV, Magalhães A, Diniz CR & De Oliveira EB (1996). Purification and properties of the thrombin-like enzyme from the venom of *Lachesis muta muta*. *International*

Journal of Biochemistry, 21: 863-871.

24. Serrano SMT, Reinhardt M, Sampaio CAM & Fink E (1995). Purification, characterization and amino acid sequence of a serine proteinase, PA-BJ, with platelet-aggregating activity from the venom of *Bothrops jararaca*. *Biochemistry*, 34: 7186-7193.
[Medline]

25. Zaganelli GL, Zaganelli MGM, Magalhães A, Diniz CR & De Lima EM (1996). Purification and characterization of a fibrinogen-clotting enzyme from the venom of Jaracucu (*Bothrops jararacussu*). *Toxicon*, 34: 807-819.
[Medline]

26. Wuster W, Thorpe RS & Porto J (1996). Systematics of the *Bothrops atrox* complex (Reptilia:Serpentes:Viperidae) in Brazil: a multivariate analysis. *Herpetologica*, 52: 263-271.

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Non-synergistic interactions between strong allosteric effectors and human embryonic and adult haemoglobins

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Abstract:

The binding of two strong allosteric effectors (2,3 Diphosphoglycerate D.P.G., and Bezafibrate, Bzf) to both adult and the three human embryonic haemoglobins, either individually or in combination, have been studied in detail. The adult protein exhibits one binding site for D.P.G and two for Bzf. When both effectors are present simultaneously their effects are simply additive. The same qualitative pattern of binding is observed in the case of the three human embryonic haemoglobins, although with different binding constants. The lack of synergism between these effectors and the different binding affinity expressed by these proteins are discussed in terms of the known amino acid sequence differences.

Keywords:

haemoglobin, embryonic, allosteric, effectors

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Kinetic characterization of a T-state of Ascaris suum phosphofructokinase with heterotropic negative cooperativity by ATP eliminated.

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The affinity analogue, 2',3'-dialdehyde ATP has been used to chemically modify the ATP-inhibitory site of Ascaris suum phosphofructokinase, thereby locking the enzyme into a less active T-state. This enzyme form has a maximum velocity that is 10% that of the native enzyme in the direction of fructose 6-phosphate (F6P) phosphorylation. The enzyme displays sigmoid saturation for the substrate fructose 6-phosphate (S0.5 (F6P) = 19 mM and nH = 2.2) at pH 6.8 and a hyperbolic saturation curve for MgATP with a Km identical to that for the native enzyme. The allosteric effectors, fructose 2,6-bisphosphate and AMP, do not affect the S0.5 for F6P but produce a slight (1.5- and 2-fold, respectively) V-type activation with Ka values (effector concentration required for half-maximal activation) of 0.40 and 0.24 mM, respectively. Their activating effects are additive and not synergistic. The kinetic mechanism for the modified enzyme is steady-state-ordered with MgATP as the first substrate and MgADP as the last product to be released from the enzyme surface. The decrease in V and V/K values for the reactants likely results from a decrease in the equilibrium constant for the isomerization of the E:MgATP binary complex, thus favoring an unisomerized form. The V and V/KF6P are pH dependent with similar pK values of about 7 on the acid side and 9.8 on the basic side. The microenvironment of the active site appears to be affected minimally as evidenced by the similarity of the pK values for the groups involved in the binding site for F6P in the modified and native enzymes.

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An allosteric switch controls the procoagulant and anticoag of thrombin.

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Abstract

Thrombin is an allosteric enzyme existing in two forms, slow and fast, that dif specificities toward synthetic and natural amide substrates. The two forms are in vivo, and the allosteric equilibrium can be affected by the binding of effectc substrates. The fast form is procoagulant because it cleaves fibrinogen with hi slow form is anticoagulant because it cleaves protein C with higher specificity thrombomodulin inhibits cleavage of fibrinogen by the fast form and promotes by the slow form. The allosteric properties of thrombin, which has targeted tw conformational states toward its two fundamental and competing roles in hem paradigmatic of a molecular strategy that is likely to be exploited by other prot coagulation cascade.

Full text